The Activity of Heparin in the Presence and Absence of Ca^{2+} Ions

The Activity of Heparin in the Presence and Absence of Ca^{2+} Ions; why the Anti-Xa Activity of LMW Heparins Is about two Times Overestimated

Dear Sir,

The activity of a heparin preparation is most easily determined relative to a heparin standard. It is also possible to express this activity in an absolute way by indicating how much a given concentration of the heparin increases the velocity of thrombin or factor Xa inactivation in plasma. This can be quantitatively expressed as the increase of the reaction constant in the reaction:

Thrombin (factor Xa) + AT III → Inactive product

For reasons that are of little importance here, the reaction constant, i.e. the decay constant of the enzyme in the plasma-medium (k_{dec}) is expressed in min^{-1} per μM of AT III in the plasma (1). This constant k_{dec} is most easily visualised as being proportional to the inverse of the half-life time of the activated clotting factor in plasma. Heparin activities can be unequivocally defined in this way and do not need to be compared to a standard; it is also independent of the method of comparison with the standard.

It is, however, not independent of the reaction conditions. Because heparins, when administered to the patient, exert their anticoagulant action in the presence of Ca^{2+}, it is natural to compare heparin activities at physiological concentrations of Ca^{2+}.

It has been found previously that the heparin dependent decay of thrombin, induced by any type of heparin, is hardly dependent on the Ca^{2+} concentration (2). This is not true for factor Xa, however, where Ca^{2+} ions produce a nearly twofold increase of the activity of unfractionated heparin (UFH), whereas it has a variable but considerably smaller effect on different low molecular weight heparins (2, 3). In this letter we should like to call attention to the important consequences of these observations for the comparison of the anti-factor Xa activity of a LMWH to that of a UFH standard.

It is common practice to compare anti-factor Xa activities in the absence of Ca^{2+} ions, for the very reason that in the presence of Ca^{2+} ions special precautions have to be taken to prevent disturbance of the measurement by activation of the clotting system. *In vivo*, however, the heparins act in the presence of Ca^{2+}, thus the activities measured *in vitro* in the presence of Ca^{2+} must be considered to have greater pharmacological relevance. With regard to the inactivation of factor Xa (*not* of thrombin), UFH is considerably suppressed in the absence of Ca^{2+}, whereas LMWH is not. This means that the anti-factor Xa activity of a LMWH, when it is compared to a UFH standard *in vitro* without Ca^{2+} ions, is measured against a shrunken yardstick. Consequently its anti-factor Xa activity is overestimated but not its anti-thrombin activity. A spuriously high ratio of anti-factor Xa activity over anti-thrombin activity results.

One might think that comparison of a LMWH to the LMWH standard would remedy this situation. This is not true as long as the declared activity of the LMWH standard itself is incorrect because it has been determined by comparison to a UFH standard in the absence of Ca^{2+}. The comparison of a LMWH to the LMWH standard may be correct and independent of the Ca^{2+} concentration, but the anti-factor Xa activity of the LMWH standard itself has been obtained by calibration against the UFH standard in the absence of Ca^{2+} ions, so that its declared activity is overrated.

Table 1 Activities of UF- and LMW heparin standards in the presence and absence of Ca^{2+} ions

	ISH	LMWHS
Activity declared (IU/mg)		
anti-thrombin	193	67
anti-factor Xa	193	168
Absolute anti-thrombin activity (at 1 μg/ml):		
$+Ca^{2+}$	13.65	6.55
$-Ca^{2+}$	13.55	6.50
Absolute anti-factor Xa activity (at 1 μg/ml):		
$+Ca^{2+}$	4.25	2.01
$-Ca^{2+}$	1.88	1.60
Activity in SIU/mg:		
anti-thrombin	13650	6550
anti-factor Xa	4250	2010

The absolute activities are expressed in $min^{-1}/\mu M$ AT III. Each value is obtained from minimally 12 determinations and the SEMs vary between 1.5 and 3%.
Abbreviations: ISH: 4th International Standard Heparin; LMWS: 1st International Low Molecular Weight Heparin Standard, IU = International Unit (= USP Unit), SIU = Standard Independent Unit.

In order to estimate the precise magnitude of the overestimation of the anti-factor Xa activity of LMWHs due to the Ca^{2+} effect, we accurately determined (SEM <3%) the absolute activities of the International Heparin Standard (IHS) and of the Low Molecular Weight Heparin Standard (11) in the presence and absence of Ca^{2+} ions (Table 1).

As with any other valid activity test, the results of the absolute activity measurements can be used to compare the potency of the preparation under study (i.e. the first international low molecular weight standard [LMWS]) to the 4th international heparin standard (IHS). The ratio of the absolute activity of the LMWHS to that of the IHS is then multiplied by the IU content of the standard (= 193 IU/mg). On the basis of the anti-factor Xa activities in the absence of Ca^{2+} of the two preparations (1.88 and 1.60 resp.) we calculate a specific anti-factor Xa activity of the LMWS of 164 IU/mg ($1.60/1.88 \times 193 = 164$). This is sufficiently close to the 168 IU/mg stated by the manufacturers to conclude that indeed the value on the label is obtained by comparing activities in the absence of Ca^{2+}. It also shows that the absolute anti-factor Xa activity estimations reflect a property that is proportional to the anti-factor Xa tests used by the manufacturers to establish the potency of the standard. We then calculated the anti-factor Xa activity of the LMW standard on the basis of the absolute anti-factor Xa activities in the presence of Ca^{2+}. In this case the activity of the LMWS was found to be 92 anti-Xa IU per mg ($2.01/4.25 \times 193 = 92$) instead of the 164 IU per mg found in the absence of Ca^{2+}. Evidently in the patient these heparins act in the presence of Ca^{2+} so the 92 IU/mg is the more relevant figure.

The reportedly high anti-factor Xa activity of LMWH preparations must be attributed to at least two causes. One is the presence of a certain amount of heparin molecules that, like pentasaccharide, have anti-factor Xa activity but are too small to have anti-thrombin activity below critical chainlength material

(BCLM, see e. g. [5]). The other is the laboratory artifact caused by the Ca^{2+} effect discussed here. Due to the Ca^{2+} effect the anti-factor Xa activity is overestimated to reach 168 IU/ml rather than 92 IU/ml. In our opinion these results show that comparison of different types of heparin to the UFH standard in the absence of Ca^{2+} leads to confusion. We do not exclude the possibility that there also exist heparin molecules with a high specific anti-factor Xa activity and a low anti-thrombin activity but our results show that such molecules are not the only, and not even the major cause of increased anti-factor Xa activity in LMWHs.

A strong argument in favour of the use of a LMWH standard follows from these observations. Comparison of LMW heparins among each other will be largely independent of Ca^{2+} effects. So if an adequately calibrated LMWH standard (i. e. in the present case 92 IU anti Xa/mg) is available, current methods that are employed in the absence of Ca^{2+} can for all practical purposes still be used to determine the activity of other LMWHs. This means that, on basis of our present results, we recant our previous opinion about the limited usefulness of a LMWH standard (6).

Additionally, it is worth our attention that 1 International Unit represents a roughly threefold higher absolute catalytic activity on thrombin decay than on factor Xa decay (Table 1). This fact easily escapes attention if the anti-thrombin and the anti-factor Xa activities of a standard preparation (UFH standard) by definition are said to be equal. This is one of the reasons to propose the use of the absolute activities for the quantisation of heparin potency. Another reason is that this opens the possibility to calculate the concentrations of active circulating heparins in terms of µg per ml plasma (5). We consequently defined a standard independent unit of heparin activity (SIU) as that amount of heparin that raises the absolute activity by 1 min^{-1} per µM of AT III in the plasma (5). In Table 1 we also give the potency of the two standards in terms of SIU. From these data it can be seen that 1,000 IUs of anti-thrombin activity represent 7.1 aIIa-SIU whereas 1,000 anti-factor Xa units represent 2.2 aXa-SIU. The shift from old to new units can be made by using these calculation coefficients.

The fact that LMWHs should be compared to an UFH standard in the presence of Ca^{2+} is nevertheless independent of the units in which heparin activities are expressed.

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Considerations about the Correct Nomenclature of Glycosaminoglycans (GAGs)

Dear Sir,

Recently you published a convient method for molecular mass determination of heparin (1). We would like to support the attempt from Dr. Nielsen to rename "low molecular weight heparin" to "low molecular mass heparin". Therefore we would like to focus on that problem and to propose to rename also the other glycosaminoglycans according to their molecular mass and their chemical structure. Mucopolysaccharides are endogenous materials with widespread origins and biological effects. The most important representatives of therapeutic use are heparins and dermatansulfates. Heparin is an important anticoagulant drug and has a molecular mass distribution between 3,000 and 30,000 Dalton (2). Several low molecular mass heparins (LMMH) have been developed in order to avoid side reactions and to improve bioavailability. Dermatansulfates have been successfully tested for their anticoagulant potential. By depolymerisation of dermatansulfates low molecular mass compounds have already been gained.

These standard or unfractionated and low molecular mass compounds are heterogeneous in their structure, degree of sulfatation, and polymerisation and the distribution of the molecular mass (3, 4). The term "glycosaminoglycan" was introduced as a biochemical classification to indicate the chief common characteristic of these and related compounds in the presence of an aminosugar. Sequencing of these anionic polysaccharides is not yet possible. But heparins, dermatansulfates and chondroitinsulfates have been cleaved to defined oligosaccharides by heparinases or chondroitinases. Thus almost all disaccharide units of the GAGs have been defined (5). The structure of major disaccharide sequences of heparin was established for example as alpha-1,4-linked L-iduronic acid 2-sulfate → glucosamine N,6-disulfate ($IdoA\text{-}2SO_3 \rightarrow GlcNSO_3\text{-}6SO_3$) (6).

The low molecular compounds are classified according to their original substances. Individual structures have not been taken into consideration to name the compounds. This may be justified because even the original material is heterogeneous. Heparin, heparan and hyaluronic acid consist of glucosamine and uronic acid. So they could be named polyglucosaminouronates (Table 1). However, dermatansulfate and chondroitinsulfates A

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